A 2-Year Dose–Response Study of Lesion Sequences during Hepatocellular Carcinogenesis in the Male B6C3F$_1$ Mouse Given the Drinking Water Chemical Dichloroacetic Acid

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Dichloroacetic acid (DCA) is carcinogenic to the B6C3F$_1$ mouse and the F344 rat. Given the carcinogenic potential of DCA in rodent liver and the known concentrations of this compound in drinking water, reliable biologically based models to reduce the uncertainty of risk assessment for human exposure to DCA are needed. Development of such models requires identification and quantification of premalignant hepatic lesions, identification of the doses at which these lesions occur, and determination of the likelihood that these lesions will progress to cancer. In this study we determined the dose response of histopathologic changes occurring in the livers of mice exposed to DCA (0.05–3.5 g/L) for 26–100 weeks. Lesions were classified as foci of cellular alteration smaller than one liver lobule (altered hepatic foci; AHF), foci of cellular alteration larger than one liver lobule (large foci of cellular alteration; LFCA), adenomas (ADs), or carcinomas (CAs). Histopathologic analysis of 598 premalignant lesions revealed that *a*) each lesion class had a predominant phenotype; *b*) AHF, LFCA, and AD demonstrated neoplastic progression with time; and *c*) independent of DCA dose and length of exposure effects, some toxic/adaptive changes in noninvolved liver were related to this neoplastic progression. A lesion sequence for carcinogenesis in male B6C3F$_1$ mouse liver has been proposed that will enable development of a biologically based mathematical model for DCA. Because all classes of premalignant lesions and CAs were found at both lower and higher doses, these data are consistent with the conclusion that nongenotoxic mechanisms, such as negative selection, are relevant to DCA carcinogenesis at lower doses where DCA genotoxicity has not been observed. *Key words:* B6C3F$_1$ mice, dichloracetic acid, drinking water disinfection by-products, hepatocarcinogenicity, histopathology, liver toxicity. *Environ Health Perspect* 111:53–64 (2003). [Online 2 December 2002]

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The reauthorization of the Safe Drinking Water Act of 1996 requires the U.S. Environmental Protection Agency (EPA) to develop a priority list of chemicals present in drinking water and to conduct research into the modes and mechanisms of action by which they produce adverse effects (Safe Drinking Water Act Amendments of 1996). Disinfection by-products are included in the priority list. The haloacetic acids, along with the trihalomethanes and haloacetonitriles, are the disinfection by-products found at the highest concentrations in drinking water after chlorine disinfection of surface waters (Krasner et al. 1989). Dichloroacetic acid (DCA) may occur in drinking water at concentrations > 100 µg/L (Uden and Miller 1983) and has median concentrations in the 15–19 µg/L range (Fair 1996; Krasner at al. 1989).

The carcinogenicity of DCA in the liver of the male and female B6C3F$_1$ mouse and the F344 male rat has been well demonstrated (Bull et al. 1990; Daniel et al. 1992; DeAngelo et al. 1991, 1996, 1999; Herren-Freund et al. 1987; Pereira 1996). The question arose whether DCA was promoting the outgrowth of initiated cells already present in the liver because the male B6C3F$_1$ mouse has a high rate of spontaneous liver tumor formation, and prior initiation with a genotoxic carcinogen was not required for DCA-induced liver tumor formation (DeAngelo et al. 1991). More recent data for the female B6C3F$_1$ mouse and for the C3H and C57BL parental strains revealed a biphasic dose–response curve for the number of carcinomas (CAs) per liver (DeAngelo 2000b; DeAngelo et al. 1996, 1999, unpublished oberservations). There was an increase in the number of CAs for the male B6C3F$_1$ and C3H mouse, strains with high spontaneous tumor rates, when animals were given 0.5 g/L DCA. In contrast, no increase in the number of CAs was seen at 0.5 g/L in animals with a low-background spontaneous CA rate (male C57BL mouse and female B6C3F$_1$ mouse), indicating an association between the background tumor incidence and the CA response at 0.5 g/L DCA. Concentrations ≥ 0.5 g/L resulted in a steeper dose–response curve. At the highest concentrations tested, 3.5–5 g/L DCA, there were no differences between strains or sexes in the CA multiplicity respective of the spontaneous tumor background rate (DeAngelo 2000b; DeAngelo et al. 1996, 1999). Although high concentrations (> 2 g/L) of DCA induced gene mutations and chromosomal damage (clastogenesis) in several *in vivo* and *in vitro* test systems (DeMarini et al. 1994; Fuscoe et al. 1995; Harrington-Brock et al. 1998; Leavitt et al. 1997), the role of genotoxicity in the DCA-induced carcinogenic process *in vivo* has not been clearly defined (Chang et al. 1992; Fox et al. 1996; Giller et al. 1997; Kopfler et al. 1985).

Much evidence has been put forth to support the concept that DCA is acting through nongenotoxic mechanisms at the lower concentrations (0.5 g/L and 1.0 g/L) that enhance liver neoplasia (International Life Sciences Institute 1997; Klaunig et al. 2000). For example, DCA treatment induced significant effects during the first 30 days of exposure, before development of hepatic lesions. Effects of DCA treatment on B6C3F$_1$ mouse liver during the first 30 days of exposure to drinking water containing either 0.5 g/L or 5.0 g/L included induction of hypertrophy (Carter et al. 1995a); alteration in nuclear size and ploidy (Carter et al. 1995a); inhibition of hepatocyte proliferation (Carter et al. 1995a; DeAngelo 2000a); and decreased apoptosis (Snyder et al. 1995). Hepatocyte hypertrophy reflected accumulation of glycogen and peroxisome proliferation (Bull et al. 1990; DeAngelo et al. 1989).

DCA also induced the formation of foci of phenotypically altered cells before the development of benign or malignant hepatic neoplasms (Bull et al. 1990; DeAngelo et al. 1991, 1996). These foci of cellular alteration were integrated into the normal architecture of the

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tissue and did not show expansive growth. Immunohistochemical techniques demonstrated that large foci of cellular alteration (LFCA; formerly called hyperplastic nodules) were putative preneoplastic lesions in the progression of DCA-induced hepatocarcinogenesis in both male $B6C3F_1$ mice and male F344 rats (Richmond et al. 1991, 1995). The presence of isolated, highly dysplasic hepatocytes in male $B6C3F_1$ mice chronically exposed to DCA suggested another direct neoplastic conversion pathway (Carter et al. 1995a, 1995b). The relevance of these preneoplastic changes for DCA-induced carcinogenesis has not been tested by statistical methods.

The potential risks of human exposure to DCA are indicated by *a*) the carcinogenic potential of DCA in rodent liver; *b*) the known concentrations of this compound in drinking water; and *c*) epidemiologic studies reporting that drinking water sources containing high concentrations of disinfection by-products are associated with an increased human cancer risk (Morris et al. 1992). However, because of the susceptibility of the untreated male $B6C3F_1$ mouse to development of hepatocellular neoplasms and the high concentrations of DCA required to increase the prevalence of CA (3–4 orders of magnitude above the concentrations in drinking water), a reliable biologically based dose–response model to reduce the uncertainty in the risk assessment for human exposure to DCA is needed (Rabinowitz et al. 2000, 2001). Such a biologically based pharmacodynamic model will relate fundamental cellular processes to the epidemiology of cancer in animal and human populations and help to extrapolate across species, from mice to humans, and from high-dose experimental conditions to low dose environmental exposures (Travis 1988). A biologically based dose–response model for DCA should include an analysis of the preneoplastic changes induced in hepatocytes by DCA and the stability of these changes; the dose at which these preneoplastic changes occur and the shape of the dose–response curve; and the likelihood that the preneoplastic changes will develop into neoplasia.

To begin addressing these questions, the present histopathologic analysis includes classification, quantification, and statistical analysis of hepatic lesions arising in male $B6C3F_1$ mice receiving DCA at doses as low as 0.05 g/L for 100 weeks and at 0.5, 1.0, 2.0, and 3.5 g/L for between 26 and 100 weeks (DeAngelo et al. 1999). As recently recommended (Harada et al. 1999), the analysis includes a separation of preneoplastic and benign hepatic lesions into subtypes as well as a grouping of subtypes. As a result of this analysis, a model for DCA-induced carcinogenesis has been developed that lends itself to testing by mathematical means (Rabinowitz et al. 2000, 2001). Histopathologic analysis of the toxic and adaptive responses to DCA in the male $B6C3F_1$ mouse liver reveals that negative selection of cells with a new state of differentiation is a probable mechanism for DCA hepatocellular carcinogenesis in this model (Farber 1990; Farber and Rubin 1991; Harada et al. 1999; Maronpot 1991).

Materials and Methods

Tissues. Tissues were from a previously published U.S. EPA study of the hepatocarcinogenicity of DCA in the male $B6C3F_1$ mouse (DeAngelo et al. 1999). In that U.S. EPA study, weanling male $B6C3F_1$ mice, 28–30 days of age, were separated into the 0 (control), 0.5, 1, 2, and 3.5 g/L DCA dose groups having 53, 55, 71, 55, and 46 mice, respectively. A second control group containing 30 mice and the 0.05 g/L DCA dose group (35 mice) were started 1 month later. Time-weighted water consumption was calculated over the interim and total dosing period by dividing the amount of water used over a particular time interval by the total weight of the mice in the cage and expressed as milliliters per kilogram per day. Multiplying the water consumption by the measured DCA concentration yielded mean daily doses (MDD) of 0, 8, 84, 168, 315, and 429 mg/kg/day (DeAngelo et al. 1999). Groups of 10 animals in each DCA dose group with the exception of 0.05 g/L DCA were euthanized at 26, 52, and 78 weeks. There were 34 unscheduled deaths. The remaining animals were euthanized between 90 and 100 weeks. All aspects of these studies were conducted in facilities certified by the American Association for Accreditation of Laboratory Animal Care in compliance with the guidelines of that association and the National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee.

Histopathology. Two blocks from each lobe and all lesions found at autopsy were preserved in 10% neutral buffered formalin for 24 hr and processed routinely. Slides were prepared and stained with hematoxylin and eosin (H&E).

Histopathologic analysis: diagnostic criteria for hepatocellular changes. We analyzed 1,355 liver specimens from 327 mice for histopathologic changes. Classification of hepatic lesions was based on the National Toxicology Program classification and nomenclature and on previously described histopathogenesis of mouse hepatocellular tumors (Frith and Ward 1979; Harada et al. 1999; Maronpot 1991; Ward 1984).

Altered hepatic foci (AHF) have been defined as histologically identifiable clones of cells within an organ differing phenotypically from the normal parenchyma (Maronpot 1991). AHF were groups of cells smaller than a liver lobule with cytologic changes (clear cell, spindle cell, dysplasia) or changes in staining characteristics. AHF did not compress the adjacent liver and might include portal triads.

Large foci of cellular alteration (LFCA) were lesions larger than a liver lobule that did not compress the adjacent liver but had alterations in architecture and staining or cellular characteristics. These lesions have previously been referred to as hyperplastic nodules (Bull et al. 1990; Carter et al. 1995b; DeAngelo et al. 1991, 1996; Richmond et al. 1991, 1995; Ward 1984). The present designation of these lesions as LFCA is to avoid confusion with non-neoplastic proliferative lesions termed "hepatocellular hyperplasia" that occur secondary to hepatic degeneration/necrosis or neoplasia (Harada et al. 1999). LFCA frequently retained portal triads.

Adenomas (ADs) showed growth by expansion resulting in displacement of portal triads and compression of adjacent normal tissue. ADs had both alterations in liver architecture and staining or cellular characteristics.

Carcinomas (CAs) were composed of cells with a high nuclear-to-cytoplasmic ratio and with nuclear pleomorphism and atypia that showed evidence of invasion into the adjacent tissue. These lesions frequently showed a trabecular pattern characteristic of mouse hepatocellular CAs. CAs that were metastatic or multinodular (CA within CA) were also found in some livers.

Tinctorial and cellular classification of preneoplastic lesions. To evaluate lesion sequence, we initially subclassified 598 premalignant hepatic lesions, according to Ward, as basophilic, eosinophilic, clear cell, or mixed cell (Frith and Ward 1979; Ward 1984). To simplify the data for statistical analysis, we grouped lesions into three primary types: eosinophilic, basophilic and/or clear cell, and dysplastic.

Eosinophilic lesions included lesions that were eosinophilic but could also have clear cells, spindle cells, or hyaline cells. Basophilic lesions were grouped with clear cell and mixed cell (i.e., mixed basophilic, eosinophilic, hyaline, and/or clear cells) lesions. This grouping was necessary because many lesions had both a basophilic and clear cell component, and a few (< 10%) had an eosinophilic or hyaline component. Lesions with foci of cells displaying nuclear pleomorphism, hyperchromasia, prominent nucleoli, irregular nuclear borders, and/or altered nuclear to cytoplasmic ratios were considered dysplastic irrespective of their tinctorial characteristics.

Because tinctorial and cytologic characterizations can be subject to error due to staining techniques and observer subjectivity, the following approaches were taken to standardize the data: *a*) all H&E stains were performed in one laboratory using the same reagents and methods; *b*) the slides were reviewed by two

observers (H.W.C. and J.H.C.) at a two-headed microscope; *c*) the observers were blinded to the treatment groups; and *d*) the slides were reviewed multiple times.

We evaluated the histologic sections of livers from treated and control animals for evidence of toxic or adaptive responses. These changes included: necrosis, glycogen accumulation (i.e., rarefaction), cytomegaly, steatosis (i.e., lipidosis), atypical nuclei, and enlarged nuclei. Zonality of the changes was noted. The histopathologic evaluation of each slide was entered into a database (Q&A; Symantec Corporation, Cupertino, CA) and the data were sorted according to DCA dose, lesion class, and subclass.

Statistical analysis. We analyzed the number of lesions per animal using log-linear Poisson regression with dose and time effects in the model (Kleinbaum et al. 1982). Linear trends were considered. At each necropsy time, the number of lesions per animal at each dose level was compared to the number of lesions per animal in the control group, again using log-linear Poisson regression. We analyzed the incidence of toxic or adaptive responses (e.g., enlarged nuclei) in mouse liver using logistic regression with dose and time effects in the model (Kleinbaum et al. 1982). Linear trends were considered. At each necropsy time, incidence of toxic or adaptive responses at each dose level was compared to the incidence of these responses in the control group using an exact chi-square analysis (Kleinbaum et al. 1982). All statistical analyses were done using SAS software (SAS Institute, Inc., Cary, NC).

Results

Quantification of premalignant lesions and their subclasses. Premalignant hepatic lesions (Figure 1) were separated into three classes based on three cellular phenotypes: eosinophilic, basophilic and/or clear cell, and dysplastic. Phenotypic diversity and heterogeneity of cell populations within hepatic lesions increased from AHF to LFCA and to AD (Table 1, Figure 2). Histopathologic analysis of 598 premalignant lesions revealed that AHF, LFCA, and ADs demonstrated neoplastic progression by the presence of foci of dysplastic cells morphologically similar to cells within hepatocellular CAs (Figure 1) and that each class of premalignant hepatic lesion had a predominant phenotype (Tables 1 and 2, Figure 2).

Of the 318 AHF observed in the random histologic sections of liver, 57% were

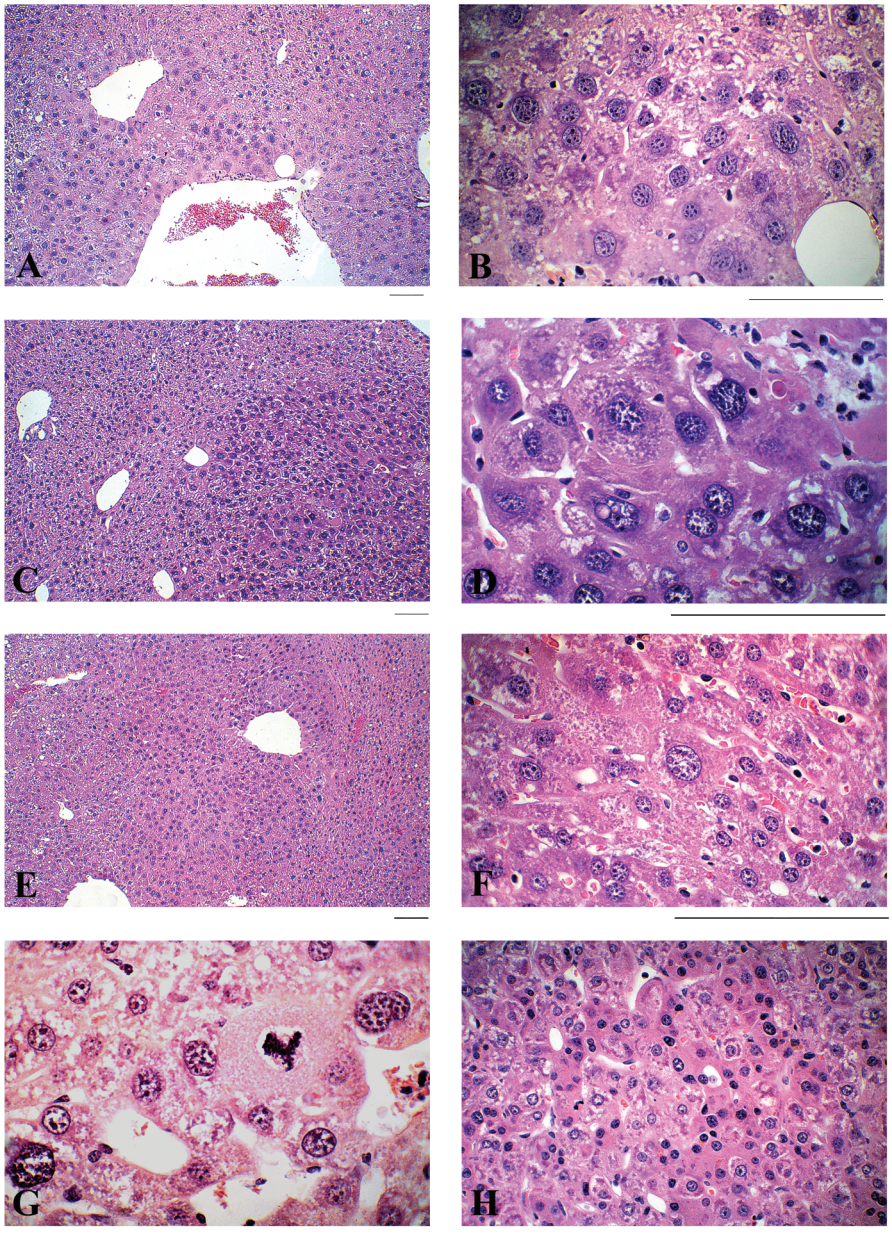

Figure 1. Neoplastic progression in B6C3F$_1$ mouse liver. (*A*) Low-power photomicrograph of an AHF in a control mouse, which is recognizable as dysplastic under higher power (magnification, 63×; bar = 100 µm). (*B*) Higher magnification of AHF in (*A*) illustrating dysplasia including nuclear enlargement, increased nuclear/cytoplasmic ratio, nuclear hyperchromasia, variation in nuclear size and shape, irregular nuclear borders, and nucleoli that are increased in size and number with irregular borders (magnification, 250×; bar = 100 µm). (*C*) LFCA in a liver from a mouse treated with 1 g/L DCA; note irregular border and lack of compression at edge (magnification, 63×; bar = 100 µm). (*D*) Higher magnification of LFCA in (*C*) illustrating a focus of dysplastic cells within the LFCA (magnification, 400×; bar = 100 µm). (*E*) Edge of a large AD from a mouse treated with 3.5 g/L DCA, demonstrating compression of adjacent parenchyma and "pushing" border of lesion (magnification, 63×; bar = 100 µm). (*F*) Higher magnification of AD in (*E*) illustrating dysplastic cells (magnification, 400×; bar = 100 µm). (*G*) Tripolar mitosis and atypical cells adjacent to normal hepatocytes in a section of normal liver from a mouse treated with 3.5 g/L DCA (magnification, 400×; bar = 100 µm). (*H*) CA from a mouse treated with 0.05 g/L DCA; note similarity between cells in this CA and the dysplastic cells found in the AHF, LFCA, AD, and normal liver illustrated in (*B*,*D*, *F*, and *G)* (magnification, 250×; bar = 100 µm).

Table 1. Subclasses of premalignant hepatic lesions shown as percent.

	Lesion type		
Cellular phenotype	AHF (n = 318)	LFCA (n = 145)	AD (n = 135)
Dys	12.6 (n = 40)	32.4 (n = 47)	23 (n = 31)
B/CC	30.8 (n = 98)	63.5 (n = 92)	51.8 (n = 70)
E	56.6 (n = 180)	4.1 (n = 6)	25.2 (n = 34)

Abbreviations: Dys, dysplastic; B/CC, basophilic and/or clear cell; E, eosinophilic. A total of 1,355 liver sections from 327 mice with 598 premalignant lesions were examined microscopically. Premalignant hepatic lesions were classified according to size and growth pattern as AHF, LFCA, or ADs and grouped according to cellular phenotype and staining characteristics. Grouping, which was for purposes of statistical analysis, combined several subclasses as shown in Figure 2.

eosinophilic, 31% were basophilic and/or clear cell, and 13% were dysplastic (Table 1, Figures 1 and 2). These small lesions were not phenotypically diverse (Figure 2).

Only 4% of the LFCA were eosinophilic (Table 1). Sixty-four percent of the LFCA were basophilic and/or clear cell. Of these basophilic and/or clear cell LFCA, 62 (67%) retained portal triads (Figure 2B). This is in contrast to AHF, where only 4/98 (4%) of the basophilic and/or clear cell altered hepatic foci had portal triads (Figure 2B). Therefore, analysis of cellular phenotype demonstrated that basophilic and/or clear cell AHF had enhanced growth potential leading to formation of LFCA. Most (89%) of the LFCA with dysplasia had a basophilic and/or clear cell component, and 25/47 (53%) of the dysplastic LFCA retained portal triads (Figure 2C), indicating that basophilic and/or clear cell LFCA retaining portal triads had malignant potential. This conclusion was strengthened by the finding of portal triads in some CAs.

Adenomas were the most phenotypically diverse premalignant lesions (Figure 2). Of the ADs, 25% were eosinophilic (Table 1). Fifty-two percent of the ADs were basophilic and/or clear cell (Table 1), but only 15/70 (21%) had portal triads (Figure 2B). The majority of ADs with areas of dysplasia or CA (68%) had a basophilic and/or clear cell component, but only 4/31 (13%) had portal triads (Figure 2C). In contrast to dysplastic LFCA, in which only 8/47 (17%) had an eosinophilic component, 10/31 (32%) of the dysplastic ADs had some eosinophilic cells.

Lesion sequence. Based on prevalence and diversity in each premalignant subclass, three lesion sequences leading to CAs in the livers of male $B6C3F_1$ mice have been proposed. Figure 3 shows the three pathways by which the premalignant hepatic lesions progressed from initiated cells to CAs based upon the histopathologic data.

In the first carcinogenic sequence, eosinophilic AHF developed into ADs and then to CAs (Figure 3). Evolution of eosinophilic AHF to eosinophilic ADs and conversion of eosinophilic ADs to CAs were infrequent events (Table 1). Although 57% (180/318) of the AHF were eosinophilic, only 25% (34/135) of the ADs were eosinophilic and without evidence of neoplastic progression. Only 7% (10/135) of the ADs had both eosinophilic cells and evidence of dysplasia.

The second carcinogenic sequence (Figure 3, Table 1) was from basophilic and/or clear cell AHF to basophilic and/or clear cell LFCA. This was the predominant sequence observed. Basophilic and/or clear cell LFCA retaining portal triads progressed to CAs directly, and basophilic and/or clear cell LFCA without portal triads progressed to ADs and then to CAs. Thirty-two percent (47/145) of the LFCA had foci of dysplasia or CA, whereas 52% (70/135) of the ADs were basophilic and/or clear cell without evidence of neoplastic progression, and 16% (21/135) of the ADs were basophilic and/or clear cell with foci of dysplasia (Table 1, Figure 2).

In the third carcinogenic sequence (Figure 3), hepatic CAs in DCA-treated animals developed from a single initiated cell (Figure 4), which, by promotion and clonal expansion, led to dysplastic AHF and then, by promotion and progression, to CAs. Thirteen percent of the AHF were associated with this lesion sequence (Table 1).

The effect of DCA treatment on the outgrowth and progression of hepatic lesions. DCA enhanced the incidence of animals with premalignant hepatic lesions (Table 2) and lesion outgrowth (lesions per animal). To determine the effect of DCA on neoplastic progression in male $B6C3F_1$ mouse liver, the number of hepatic lesions per animal was statistically evaluated using log-linear Poisson regression analysis with time and dose effects in the model (Tables 3 and 4) (Kleinbaum et al. 1982). At each time interval, the number of lesions per animal at each dose level was compared to the number of lesions per animal in the control group using log-linear Poisson regression.

DCA (0.05–3.5 g/L) increased the number of lesions per animal relative to animals receiving distilled water and shortened the time to development of all classes of hepatic lesions (Figures 5 and 6, Table 3). Figure 5 illustrates the dose response to five concentrations of

Table 2. Number of lesions/number of animals in group and incidence of premalignant hepatic lesions (% animals) at given doses of DCA and overall.

Lesion class, subclass	Control	0.05 g/L	0.5 g/L	1.0 g/L	2.0 g/L	3.5 g/L	Overall
AHF							
Dys	4/80 (5)	1/33 (3)	3/55 (5.5)	7/65 (10.8)	9/51 (17.6)	5/43 (11.6)	29/327 (8.9)
B/CC	3/80 (3.8)	1/33 (3)	2/55 (3.6)	12/65 (18.5)	17/51 (33.3)	12/43 (27.9)	47/327 (14.4)
E	6/80 (7.5)	6/33 (18.2)	18/55 (32.7)	22/65 (33.8)	19/51 (37.3)	20/43 (46.5)	91/327 (27.8)
LFCA							
Dys	1/80 (1.2)	2/33 (6.1)	7/55 (12.7)	14/65 (21.5)	8/51 (15.7)	7/43 (16.3)	39/327 (11.9)
B/CC	4/80 (5)	1/33 (3)	7/55 (12.7)	17/65 (26.2)	16/51 (31.4)	8/43 (18.6)	53/327 (16.2)
E	0/80 (0)	0/33 (0)	0/55 (0)	1/65 (1.5)	1/51 (2)	2/43 (4.7)	4/327 (1.2)
ADs							
Dys	5/80 (6.2)	3/33 (9.1)	4/55 (7.3)	7/65 (10.8)	2/51 (3.9)	7/43 (16.3)	28/327 (8.6)
B/CC	7/80 (8.8)	10/33 (30.3)	2/55 (3.6)	9/65 (13.8)	11/51 (21.6)	18/43 (41.9)	57/327 (17.4)
E	2/80 (2.5)	0/33 (0)	2/55 (3.6)	6/65 (9.3)	7/51 (13.7)	10/43 (23.3)	27/327 (8.3)

Abbreviations: B/CC, basophilic and/or clear cell; Dys, Dysplastic; E, eosinophilic. Controls received 0 g/L DCA. The results are pooled over all lengths of exposure to illustrate the effect of dose.

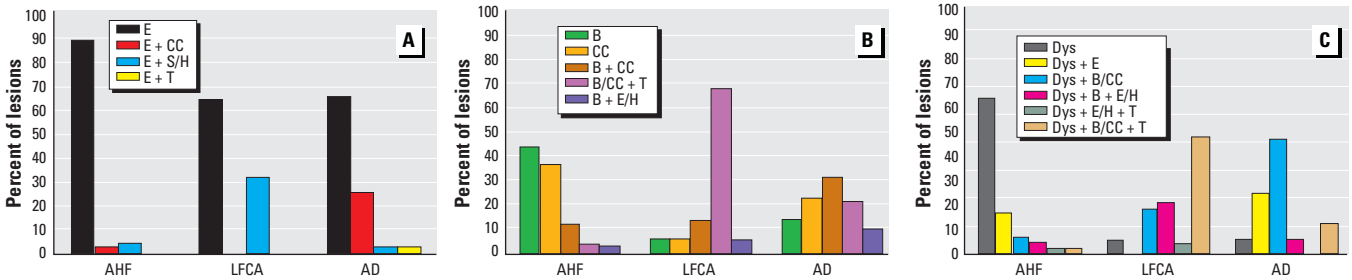

Figure 2. Phenotypic diversity and heterogeneity of cell populations within the three major subclasses of premalignant hepatic lesions, which increased with neoplastic progression. Abbreviations: Dys, dysplastic; B, basophilic; CC, clear cell; E, eosinophilic; H, hyaline cells; S, spindle cell changes, T, portal triads. (*A*) Eosinophilic lesions included 180 AHF, 6 LFCA, and 34 ADs. (*B*) B/CC lesions included 98 AHF, 92 LFCA, and 70 ADs. (*C*) Dys lesions included 40 AHF, 47 LFCA, and 31 ADs.

DCA relative to controls at 100 weeks. Figure 6 compares hepatic lesion development with length of exposure (time) up to 2 years in low dose (0.5 g/L and 1.0 g/L) and high dose (2.0 and 3.5 g/L) animals. Separation into high and low doses was based on previously reported CA incidences (DeAngelo et al. 1999) and on this independent review of the slides (Figure 5D). Table 3 gives the *p*-values for the DCA dose and time effects. Poisson regression analysis of the development of hepatic lesions with DCA dose and length of exposure indicated that all hepatic lesions were significantly related to either DCA dose or length of exposure in male $B6C3F_1$ mouse liver.

DCA dose effects. All classes of hepatic lesions were found in groups of animals receiving drinking water containing 0–3.5 g/L DCA (Table 2, Figure 5). Although this analysis could not distinguish between spontaneously arising lesions and additional lesions of the same type induced by DCA, only lesions of the kind that were found spontaneously in control liver were found in increased numbers in animals receiving DCA. The only subclass of lesion not found in control animals was eosinophilic LFCA, and DCA did not enhance the outgrowth of these lesions (Table 4).

Development of eosinophilic, basophilic and/or clear cell and dysplastic AHF was significantly related to DCA dose at 100 weeks and overall adjusted for time (Figure 5A, Table 3). Eosinophilic AHF were the most frequently found lesions at 100 weeks (Figure 5). Development of eosinophilic AHF was marginally related to DCA dose at 52 weeks and highly correlated to DCA dose at 78 and 100 weeks (Figures 5A and 6A, Table 3). Poisson regression analysis indicated three significant differences in the number of AHF per animal in DCA-treated animals relative to controls: *a*) significantly more eosinophilic AHF were found in mice receiving 0.5, 1.0, 2.0, and 3.5 g/L DCA; *b*) significantly more basophilic/clear cell AHF were found in mice receiving 1.0, 2.0, and 3.5 g/L DCA; and *c*) the number of dysplastic AHF was significantly different only in mice receiving 2.0 g/L DCA.

Basophilic and/or clear cell LFCA were the predominant subclass found at all DCA dose levels and in controls. The number per animal was highly related to DCA dose at 100 weeks and overall adjusted for time (Figure 5B, Table 3). The number of dysplastic LFCA per animal was also highly related to the DCA dose at 100 weeks (Table 3). The number of both basophilic and dysplastic LFCA per animal increased rapidly from 0 to 1.0 g/L DCA and then plateaued between 1.0 and 3.5 g/L. In mice receiving 0.05, 1.0, 2.0, or 3.5 g/L DCA, both the number of basophilic and/or clear cell and the number of dysplastic LFCA per animal were significantly different from controls.

ADs were the most frequently occurring lesions in control mice. The number of basophilic and/or clear cell ADs per animal was significantly related to dose at 52 and 100 weeks and overall adjusted for time (Figure 5C, Table 3). Development of eosinophilic ADs was significantly related to dose at 52, 78, and 100 weeks and overall (Table 3). Dysplastic ADs were related to dose only at 52 weeks. The number of basophilic ADs per mouse differed significantly from controls in animals receiving 0.05, 2.0, and 3.5 g/L DCA, and the number of eosinophilic ADs per mouse differed significantly from controls in animals receiving 2.0 or 3.5 g/L DCA.

As previously reported, development of CAs in DCA-treated mice was highly correlated with both DCA dose and length of exposure ($p = 0.0001$) (DeAngelo et al. 1999). This independent review of slides from that study indicated that the number of CAs per animal was significantly related to dose at 78 and 100 weeks and overall adjusted for time (Figures 5D and 6D, Table 3). This is consistent with the evolution of CAs from the dysplastic ADs

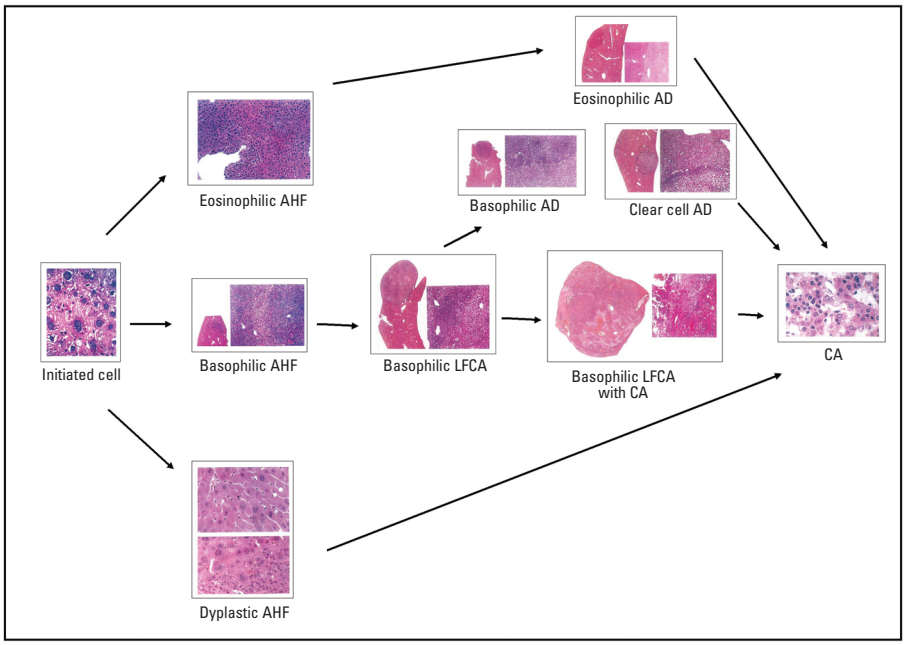

Figure 3. Proposed lesion sequences in male $B6C3F_1$ mouse liver based on histopathologic analysis of frequency of hepatic lesions found in 327 male $B6C3F_1$ mice (Table 1). The first carcinogenic sequence is from an initiated cell to an eosinophilic AHF, which progresses to an AD and then to a CA. The second carcinogenic sequence is from an initiated cell to a basophilic and/or clear cell AHF, which because of a selective growth advantage develops into basophilic and/or clear cell LFCA. In this model, basophilic and/or clear cell LFCA with portal triads progress to CAs directly and basophilic and/or clear cell LFCA without portal triads progress to ADs and then to CAs. The third carcinogenic sequence is from a single initiated cell, which develops into a dysplastic AHF by clonal expansion and then to a CA.

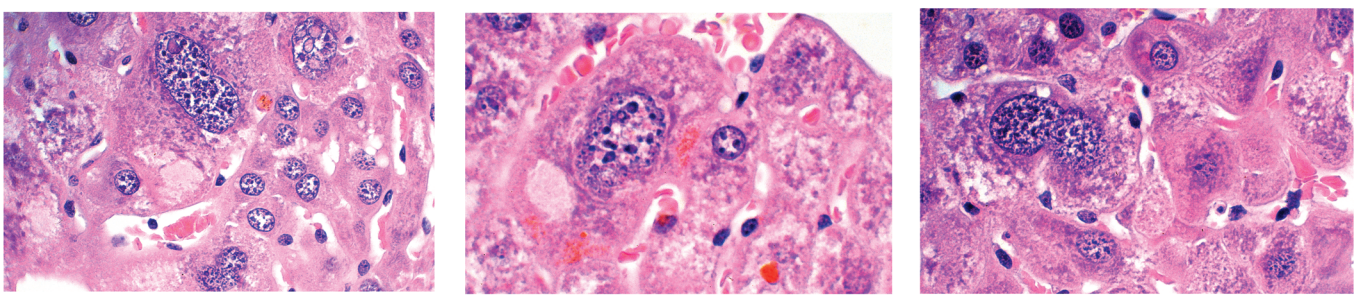

Figure 4. Examples of atypical cells found in livers of $B6C3F_1$ mice. Incidence of atypical cells in non-involved normal liver was highly associated with the number of dysplastic LFCA, dysplastic ADs, and CAs per mouse.

seen at 52 weeks. Overall, the number of CAs per animal was significantly different from controls in mice receiving 0.5, 1.0, 2.0, and 3.5 g/L DCA.

Effect of length of DCA exposure. Both AHF and ADs were found in DCA-treated animals at 26 weeks. Basophilic and dysplastic LFCA and CAs were found at 52 weeks, consistent with their evolution from these earlier appearing lesions.

Development of basophilic and/or clear cell AHF was significantly related to the length of DCA exposure between 26 and 100 weeks in animals receiving 1.0, 2.0, or 3.5 g/L DCA (Figures 5A and 6A, Table 4). Eosinophilic AHF were related to length of DCA exposure in animals receiving 0.5, 1.0, 2.0, or 3.5 g/L DCA (Table 4). The number of basophilic and eosinophilic AHF increased approximately 3-fold between 52 and 100 weeks. Consistent with the later appearance of dysplastic AHF, there was a trend for a relationship between dysplastic AHF and DCA dose at 78 weeks, and a significant relationship with DCA dose at 100 weeks (Table 3). The relationship between dysplastic AHF and length of exposure was significant only in mice receiving 2.0 g/L DCA (Table 4).

Development of basophilic and/or clear cell LFCA was related to the length of exposure to drinking water containing 1.0, 2.0, or 3.5 g/L DCA, and a trend was found at 0.5 g/L (Table 4). The number of dysplastic LFCA per animal was related to the length of DCA exposure at 1.0, 3.5, and 0.5 g/L, where a trend was found (Figures 5B and 6B, Table 4). Overall, the development of both basophilic and/or clear cell and dysplastic LFCA was highly related to both dose and length of exposure (Tables 3 and 4).

The number of basophilic and/or clear cell ADs per animal was significantly related to the length of exposure in mice receiving 2.0 g/L DCA (Table 4). Poisson regression analysis indicated that when all doses and exposure lengths were considered, development of basophilic and/or clear cell, eosinophilic, and dysplastic ADs were related to length of exposure (Table 4, Figures 5C and 6C).

The number of CAs per animal was significantly related to length of exposure at 1.0, 2.0, and 3.5 g/L DCA (Figures 5D and 6D, Table 4).

Toxic or adaptive responses in the livers of DCA treated mice. DeAngelo et al. (1999) recently reported that the hepatocellular CA incidence and multiplicity in these DCA-treated mice were a function of mean daily dose and that there was a significant trend for increasing absolute and relative liver weights with dose throughout the exposure period. Here, the incidence of toxic or adaptive responses in the livers of these mice was evaluated using logistic regression to determine if early toxic or adaptive hepatic morphologic changes were associated with DCA-induced neoplastic progression and hepatocellular carcinogenesis. Table 5 presents the overall incidence of toxic or adaptive responses, including hepatocellular CA, in relation to DCA dose.

Necrosis. Necrosis was found in 11.3% of the animals in the study and was the least prevalent toxic or adaptive response (Table 5). At 26 weeks exposure to 3.5, 2.0, or 1.0 g/L DCA, focal necrosis was present in 50, 50, and 80% of the livers, respectively. These small areas of necrotic hepatocytes were distributed in the livers without regard to liver architecture. Three independent reviews of slides from these animals (DeAngelo et al. 1999; International Life Sciences Institute 1997; and this report) found necrosis ranging from single-cell necrosis to small clusters of necrotic hepatocytes, and to occasional panlobular coagulative necrosis in mice receiving 1.0–3.5 g/L DCA for 26 weeks. At 26 weeks, the incidence of necrosis in mice receiving 1.0, 2.0, or 3.5 g/L DCA was significantly different from that in controls (p = 0.0071, 0.0325, and 0.0325, respectively). Focal necrosis was not present at 26 weeks in animals exposed to water or to 0.5 g/L DCA. The incidence of necrosis in treated mice did not differ statistically from controls at 52 or 78 weeks. However, necrosis was significantly different from controls after 100 weeks of treatment with 3.5 g/L DCA (p = 0.0002). Overall, necrosis was negatively related to length of exposure and positively related to DCA dose (Tables 6 and 7). These results are consistent with the findings of two other pathologists (DeAngelo et al. 1999; International Life Sciences Institute 1997) and show that necrosis was an early and transitory response.

Rarefaction/glycogen accumulation. Glycogen accumulation as evidenced by clear cell changes and diffuse rarefaction (Sanchez and Bull 1990) was present in 11.9% of the animals (Table 5). At 26 weeks and 78 weeks of DCA treatment, clear cell changes were found focally in the livers; whereas at 52 weeks, a diffuse rarefaction was observed. The periodic acid Schiff (PAS) reaction was negative due to dissolution of glycogen in the aqueous fixative. Rarefaction was not found in controls and was dose dependent. Animals receiving 1.0, 2.0, or 3.5 g/L DCA for 52 weeks differed significantly from controls (p = 0.0031, 0.0325, and 0.0007, respectively);

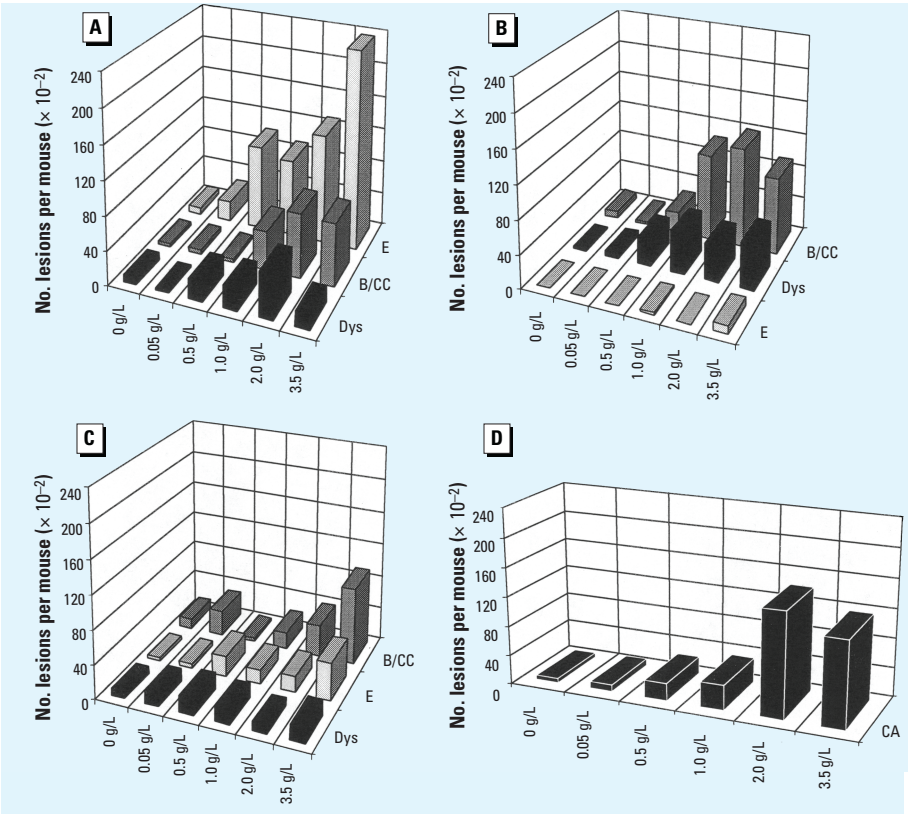

Figure 5. DCA dose response at 100 weeks in B6C3F$_1$ male mice given drinking water containing 0–3.5 g/L DCA. Lesions were classified as (*A*) AHF, (*B*) LFCA, (*C*) ADs, or (*D*) CAs and subclassified into three major groups: eosinophilic (E), basophilic and/or clear cell (B/CC), and dysplastic (Dys). Poisson regression analysis of the development of hepatic lesions with DCA dose indicated that development of B/CC, E, and Dys AHF; B/CC and Dys LFCA, B/CC, E, and Dys ADs; and CAs, was significantly related to DCA exposure in male B6C3F$_1$ mouse liver.

however, glycogen accumulation was found only in the periportal hepatocytes in mice given 0.5 g/L DCA. Focal cells did not have evidence of glycogen accumulation. Glycogen accumulation was not seen at 100 weeks and was negatively correlated with length of exposure overall adjusted for dose (Table 7).

Cytomegaly. Enlargement of hepatocytes (cytomegaly) was found in 17.4% of the animals (Table 5). Consistent with previous short-term studies (Carter et al. 1995a), cytomegaly was present in 90–100% of the livers of mice given 1.0–3.5 g/L DCA for 26 weeks. When DCA dose groups were combined over length of exposure, the incidence of cytomegaly was significantly different from controls in mice receiving 1.0, 2.0, and 3.5 g/L DCA ($p \leq 0.0001$). Overall, cytomegaly was positively related to DCA dose and negatively related to length of exposure (Tables 6 and 7).

Steatosis. Accumulation of lipid droplets (steatosis, lipidosis) was present in some hepatocytes from most groups of mice and in 24.2% of the animals overall (Table 5). The incidence of steatosis was negatively correlated with dose, reflecting the hypolipidemic effects of DCA (Stacpoole et al. 1978). There were no incidences of steatosis in six of the eight groups of animals receiving 2.0 and 3.5 g/L DCA, and this was significantly different overall from controls ($p = 0.0001$, and 0.0094, respectively, see Table 5). The incidence of steatosis was positively correlated with age and body weight in controls.

Atypical nuclei. Overall, atypical nuclei were found in 40.7% of the animals (Table 5, Figure 4). Atypical nuclei had one or more of the following characteristics: variable, irregular, misshapen, enlarged relative to the cytoplasm, and/or hyperchromatic. When nuclei had three or more of these characteristics, they were considered dysplastic. However, atypical and dysplastic cells were combined in this analysis. By 100 weeks exposure, atypical nuclei were seen in 85, 90.5, and 86% of the livers from animals exposed to 3.5, 2.0, and 1.0 g/L DCA, respectively, and also in 56, 33, and 32% of the livers from animals receiving 0.5 or 0.05 g/L DCA and in controls, respectively. Overall, the incidence of atypical nuclei in liver was significantly related to both length of exposure and DCA dose (Tables 6 and 7). The combined data indicated that the incidence of atypical nuclei in non-involved liver differed significantly from controls in mice receiving 1.0, 2.0, or 3.5 g/L DCA ($p = 0.0001$, 0.0001, and 0.0079, respectively).

Enlarged nuclei. Enlarged nuclei were present in livers of animals sacrificed throughout the study and in both control and treated animals (Table 5). Nuclear enlargement was related to DCA dose at 100 weeks but not overall adjusted for time (Table 6). This karyomegaly was zonal, being prominent near the central veins. The incidence of enlarged nuclei was related to the length of DCA exposure in animals receiving 0.5, 1.0, 2.0, or 3.5 g/L DCA (Table 7).

Adaptive changes related to neoplastic progression in DCA-induced hepatocellular carcinogenesis. Logistic regression analysis adjusting for DCA dose and length of exposure was used to evaluate the association between development of dysplasia or CA and toxic or

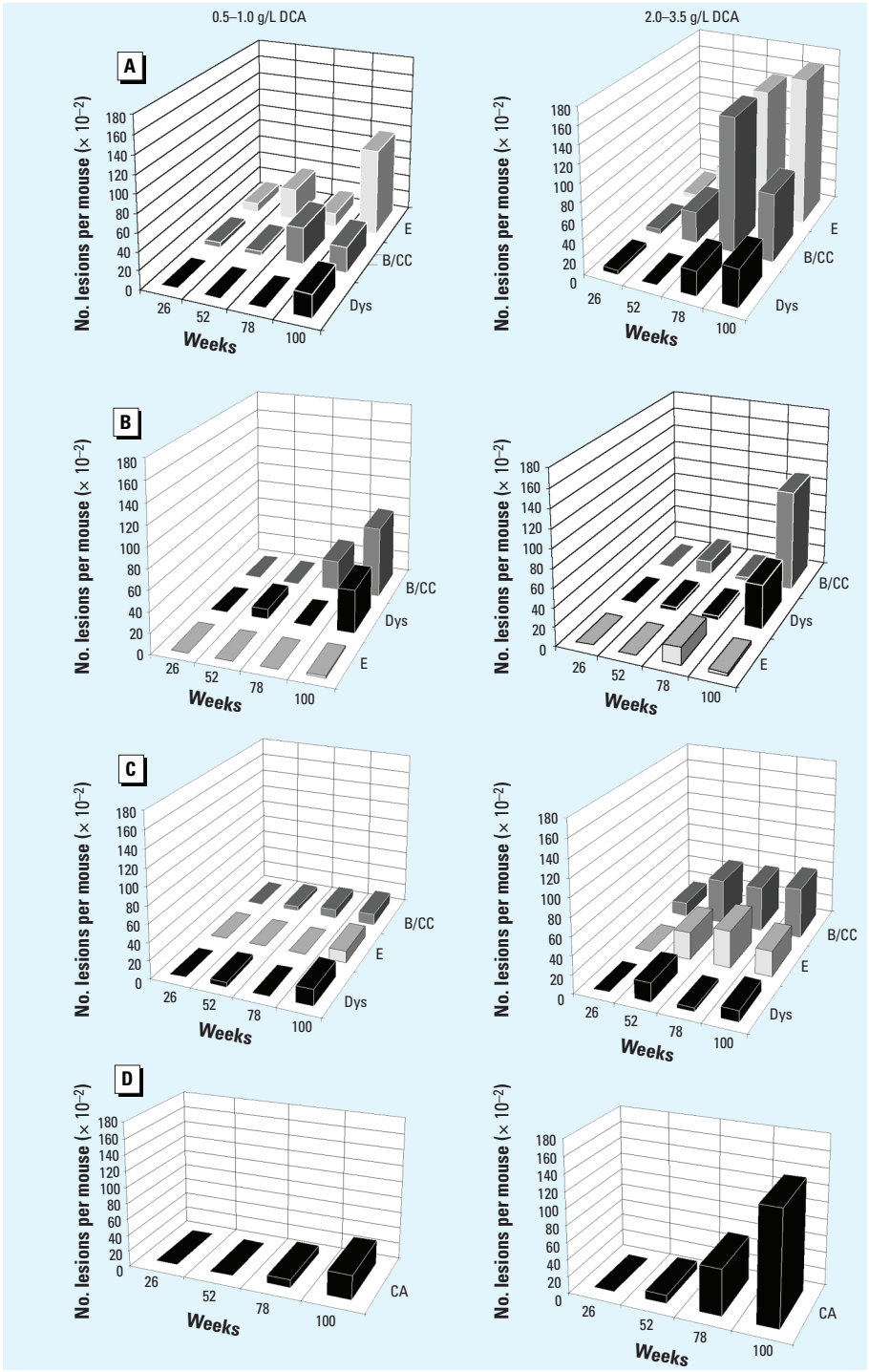

Figure 6. Development of hepatic lesions at 26, 52, 78, or 100 weeks in male B6C3F$_1$ mice exposed to drinking water containing low doses (0.5–1.0 g/L; left) and high doses (2.0–3.5 g/L; right) of DCA. Separation into low and high doses was based on previously reported CA incidences (DeAngelo et al. 1999) and the present data (Figure 5D). Lesions were classified as AHF (*A*), LFCA (*B*), ADs (*C*), or CAs (*D*). Poisson regression analysis of the development of hepatic lesions with length of exposure to DCA indicated that development of basophilic and/or clear cell (B/CC), eosinophilic (E), and dysplastic (Dys) AHF; B/CC and Dys LFCA; B/CC, E, and Dys ADs; and CAs, was significantly related to DCA exposure in male B6C3F$_1$ mouse liver. The effect of length of exposure on development of hepatic lesions was seen at both low and high doses of DCA.

adaptive changes. The occurrence of dysplastic AHF was not associated with any toxic or adaptive response. In contrast, dysplastic LFCA were significantly related to the presence of zonal changes, enlarged nuclei, and atypical nuclei in the adjacent non-involved liver (p = 0.0002, 0.0025, and 0.0001, respectively). Steatosis was negatively related to dysplastic LFCA. Dysplastic LFCA were not related to necrosis (p = 0.2386), indicating that these lesions do not represent compensatory, regenerative, or reparative hyperplasia in this model.

Nuclear atypia in non-involved hepatocytes and glycogen accumulation were associated with dysplastic ADs (p = 0.0045 and 0.0074, respectively). Necrosis was not related to occurrence of dysplastic ADs (p = 0.5027).

As with dysplastic LFCA, CAs were negatively associated with steatosis (p = 0.0020). Similar to dysplastic ADs and dysplastic LFCA, CAs were associated with atypical nuclei in the adjacent liver (p = 0.0136). Necrosis was of borderline significance in relation to the presence of hepatocellular CAs (p = 0.0553). As necrosis was not associated with development of dysplastic LFCA or ADs, this may indicate an effect of necrosis on the development of CAs directly from dysplastic foci (the third lesion sequence).

In summary, some toxic or adaptive responses associated with DCA exposure in non-involved male B6C3F$_1$ mouse livers were related to the neoplastic progression of LFCA and ADs and to the occurrence of hepatocellular CAs even after adjusting for dose and length of exposure effects. The data (Table 7) demonstrating a negative correlation between some of these responses (e.g., glycogen accumulation) and length of DCA exposure indicate adaptation in the livers of mice exposed to DCA and are consistent with the hypothesis that hepatocellular carcinogenesis in this model is associated with negative selection for cells with a new state of differentiation (Maronpot 1991) and progressive adaptation and selection within premalignant hepatic lesions (Carter et al. 2001a, 2001b).

Table 3. Log-linear Poisson regression analysis (p-values) of lesions per animal with effects of DCA dose: at sequential intervals of DCA exposure and overall adjusted for time (Overall adj).

Lesion class, subclass	26 weeks	52 weeks	78 weeks	100 weeks	Overall adj
AHF					
Dys	NS	—	0.0722[a]	0.0022	0.0004
B/CC	NS	0.0294	0.0006	0.0001	0.0001
E	NS	0.0523[a]	0.0001	0.0001	0.0001
LFCA					
Dys	—	NS	—	0.0001	0.0001
B/CC	—	NS	NS	0.0001	0.0001
E	—	—	NS	0.0692[a]	0.0081
AD					
Dys	—	0.0236	NS	NS	NS
B/CC	—	0.0007	0.0614[a]	0.0001	0.0001
E	—	0.0131	0.0081	0.0001	0.0001
CA	—	NS	0.0015	0.0001	0.0001

Abbreviations: B/CC, basophilic and/or clear cell; Dys, Dysplastic; E, eosinophilic; NS, not significant. Linear trends were considered in the analysis. Interim sacrifices included 10 animals/group with variable numbers of animals at the 100-week sacrifice.
[a] p-Values with marginal significance.

Table 4. Log-linear Poisson regression analysis (p-value) of lesions per animal with length of exposure at given doses of DCA and overall adjusted for dose (Overall adj).

Lesion class, subclass	Control	0.5 g/L	1.0 g/L	2.0 g/L	3.5 g/L	Overall adj
AHF						
Dys	NS	—	—	0.0120	NS	0.0003
B/CC	NS	NS	0.0264	0.0080	0.0118	0.0001
E	NS	0.0130	0.0024	0.0001	0.0001	0.0001
LFCA						
Dys	—	0.0676[a]	0.0235	—	0.0247	0.0001
B/CC	—	0.0855[a]	0.0009	0.0006	0.0067	0.0001
E	—	—	—	NS	NS	NS
AD						
Dys	NS	—	NS	—	NS	0.0072
B/CC	NS	NS	NS	0.0403	NS	0.0006
E	—	—	—	NS	0.0605[a]	0.0028
CA	—	—	0.0440	0.0001	0.0003	0.0001

Abbreviations: B/CC, basophilic and/or clear cell; Dys, Dysplastic; E, eosinophilic; NS, not significant. Controls received 0 g/L DCA. Linear trends were considered in the analysis. Interim sacrifices included 10 animals/group with variable numbers of animals at the 100-week sacrifice.
[a] p-Values with marginal significance.

Discussion

Given the carcinogenic potential of DCA in rodent liver (Bull et al. 1990; Daniel et al. 1992; DeAngelo et al. 1991, 1996, 1999; Herren-Freund et al. 1987; Pereira 1996) and the known concentrations of this compound in drinking water (Fair 1996; Krasner et al. 1989; Uden and Miller 1983), reliable biologically based models to reduce the uncertainty of risk assessment for human exposure to DCA are needed. Development of such models requires identification and quantification of premalignant hepatic lesions, identification of the doses at which these lesions occur, and determination of the likelihood that these lesions will progress to cancer. To address these questions, histopathologic analysis was conducted for livers and hepatic lesions arising in B6C3F$_1$ male mice receiving DCA doses as low as 0.05 g/L for 100 weeks, and DCA at 0.5, 1.0, 2.0, and 3.5 g/L for between 26 and 100 weeks (DeAngelo et al. 1999). Nongenotoxic versus potential genotoxic effects of DCA can be separated based on low dose (0.05–1.0 g/L) or low concentrations, where genotoxicity has not been demonstrated, versus high dose (2–3.5 g/L) or high concentrations, where gene mutations and clastogenesis has been observed in several test systems (Chang et al. 1992; DeMarini et al. 1994; Ferreia-Gonzalez et al. 1995; Fuscoe et al. 1995; Harrington-Brock et al. 1998; Leavitt et al. 1997) (Table 8). This analysis suggested a model for DCA-induced carcinogenesis, involving three lesion sequences, that lends itself to testing by mathematical means (Rabinowitz et al. 2000, 2001).

The liver of the male B6C3F$_1$ mouse is highly susceptible to the development of hepatocellular neoplasms and undergoes a spectrum of premalignant, benign, and malignant lesions during its lifetime (Harada et al. 1999), suggesting the presence of a population of initiated cells in the livers of young mice. With the exception of eosinophilic LFCA, all of the premalignant lesions seen in the livers from DCA-treated animals in this study were seen also in untreated animals, though in markedly lower numbers, indicating that DCA enhanced processes otherwise occurring during the aging process in B6C3F$_1$ mice.

Diagnostic criteria for identifying classes of preneoplastic and neoplastic hepatocellular lesions in mouse liver have been described (Frith and Ward 1979; Harada et al. 1999; Maronpot 1991; Ward 1984). Bannasch (1986) defined preneoplasia as being a phenotypically altered cell population that has no obvious neoplastic nature but has a high probability of progressing to a benign or malignant neoplasm. Differences in phenotype based on cytological features and tinctorial properties in H&E-stained histologic sections at the light microscopic level reflect

structural differences in cellular organization at the subcellular level such as increases in smooth endoplasmic reticulum and/or mitochondria (eosinophilia); increases in rough endoplasmic reticulum and/or free ribosomes (basophilia); accumulation of glycogen and/or lipid (clear cells); and changes in ploidy (enlarged nuclei) (Harada et al. 1999). Such changes in subcellular organization reflect changes in cell function (i.e., differentiation). Carcinogenesis can be thought of as resulting in a new state of differentiation that gives the new phenotype a selective growth advantage (Farber 1990; Farber and Rubin 1991), or as a form of toxicity where cells achieve a different steady state from normal and do not respond to normal homeostatic mechanisms (Maronpot 1991). Therefore, phenotypic changes in preneoplastic and neoplastic lesions during carcinogenesis should reveal information about the carcinogenic process induced by DCA.

The lesion sequences proposed here were based on the histopathologic evaluation of essentially two-dimensional (5 μm) histologic sections. Further sectioning of paraffin blocks indicated that AHF were not always isolated structures. For example, multiple altered foci in one section coalesced at a deeper level to appear as LFCA or ADs, and small AHF disappeared, became larger, or remained the same size at deeper levels. Acid/base staining characteristics also could vary with section level. Branching of AHF was proven previously by three-dimensional reconstruction (Imaida et al. 1989; Ito et al. 1989). These observations indicated that some premalignant hepatic lesions represent a continuum in space and time, and emphasized the fact that the probability of observing a given type of lesion in a random section of liver depends upon the frequency, size and shape of the lesion.

Despite these limitations, identification and quantification of phenotypes of hepatocellular lesions in the $B6C3F_1$ male mouse revealed three lesion sequences during mouse liver carcinogenesis (Figure 3). The first lesion sequence was from eosinophilic AHF, the most frequent lesion observed in this study, representing 30% (n = 180) of the 598 lesions examined (Table 1). The number of eosinophilic AHF per animal was significantly different from controls at both low and high doses of DCA and was significantly related to DCA dose at 100 weeks. Thus, DCA increased the number of AHF with a predominance of smooth endoplasmic reticulum and mitochondria. DCA did not promote clonal expansion of eosinophilic AHF by itself, as indicated by the facts that eosinophilic LFCA were rare in DCA treated animals (n = 6; Table 1), were not related to length of exposure, and were only marginally related to dose at 100 weeks (Tables 3 and 4). Some eosinophilic AHF developed into eosinophilic ADs directly, as shown by the data indicating that 34/135 (25%) of the ADs were eosinophilic (Table 1). The number of eosinophilic ADs per mouse differed significantly from controls in animals receiving the highest doses of DCA (2 or 3.5 g/L). Seven percent (10/135) of the ADs were eosinophilic with dysplasia or CA, illustrating that eosinophilic ADs had malignant potential.

The discrepancy between the number of eosinophilic AHF and the number of CAs observed indicated the failure of DCA alone to promote the neoplastic progression of most eosinophilic AHF in the male $B6C3F_1$ mouse except at high doses. Pereira (1996) also found that DCA induced primarily eosinophilic AHF in the livers of female mice in a dose-dependent manner. We report that 56.6% of the AHF in male $B6C3F_1$ mice were eosinophilic (Table 1), and Pereira (1996) reported that 57.2, 81.8, and 96.7% of the AHF found in female $B6C3F_1$ mice were eosinophilic in animals given DCA at 2.0, 6.67, and 20.0 mmole/L, respectively. However, in contrast to our findings in the male, where only 25.2% of the ADs were eosinophilic (Table 1), 90–100% of the neoplasms (ADs and CAs) in the female were eosinophilic (Pereira, 1996). This is not surprising because untreated male and female $B6C3F_1$ mice differ in the incidence of hepatocellular lesions and in AHF phenotypic distribution (Harada et al. 1999). The incidence of AHF, ADs, and CAs is higher in male versus female $B6C3F_1$ mice from 58 weeks until 168 weeks of age, and this difference is greatest between 84 and 109 weeks when the incidence of AHF, ADs, and CAs is 3.9-, 2.3-, and 4.5-fold higher, respectively, in male versus female controls (Harada et al. 1999). The incidence of mixed cell, clear cell, basophilic, or eosinophilic AHF ranges from 2.0 to 4.8% in female control $B6C3F_1$ mice, whereas in the male controls the incidence of clear cell foci is 12.9%, that of eosinophilic foci is 12.0% while basophilic foci and mixed cell foci each have a 3.6% incidence (Harada et al. 1999).

The second carcinogenic sequence was from basophilic and/or clear cell AHF that grew into basophilic and/or clear cell LFCA and progressed to CAs directly. Alternatively, the basophilic and/or clear cell AHF became basophilic and/or clear cell LFCA before progressing to ADs or to CAs. This was the predominant sequence found in this study (Figure 3). The combined total of basophilic and/or clear cell LFCA and ADs was greater than the number of basophilic and/or clear cell

Table 5. Number of animals with response/number of animals in group and incidence of toxic or adaptive responses (% animals) at given doses of DCA and overall.

Toxic/adaptive response	Control	0.05 g/L	0.5 g/L	1.0 g/L	2.0 g/L	3.5 g/L	Overall
Necrosis	2/80 (2.5)	2/33 (6.1)	1/55 (1.8)	13/65 (20)	6/51 (11.8)	13/43 (30.2)	37/327 (11.3)
Glycogen	3/80 (3.8)	0/33 (0)	11/55 (20)	7/65 (10.8)	6/51 (11.8)	12/43 (27.9)	39/327 (11.9)
Cytomegaly	1/80 (1.2)	0/33 (0)	0/55 (0)	20/65 (30.8)	21/51 (41.2)	15/43 (34.9)	57/327 (17.4)
CA	6/80 (7.5)	5/33 (15.2)	6/55 (10.9)	13/65 (20)	20/51 (39.2)	16/43 (37.2)	66/327 (20.2)
Steatosis	21/80 (26.3)	22/33 (66.7)	19/55 (34.5)	14/65 (21.5)	0/51 (0)	3/43 (7)	79/327 (24.2)
Zonal change	9/80 (11.2)	0/33 (0)	22/55 (40)	39/65 (60)	25/51 (49)	13/43 (30.2)	108/327 (33)
Atypical nuclei	18/80 (22.5)	11/33 (33.3)	18/55 (32.7)	36/65 (55.4)	30/51 (58.8)	20/43 (46.5)	133/327 (40.7)
Enlarged nuclei	33/80 (41.2)	13/33 (39.4)	30/55 (54.5)	36/65 (55.4)	23/51 (45.1)	18/43 (41.9)	153/327 (46.8)

Controls received 0 g/L DCA. The results were pooled over all lengths of exposure to illustrate the effect of dose.

Table 6. Logistic regression analysis (p-values) of incidence of toxic or adaptive responses with effects of DCA dose at sequential intervals of exposure and overall adjusted for time (Overall adj).

Toxic/adaptive response	26 weeks	52 weeks	78 weeks	100 weeks	Overall adj
Necrosis	0.0192	—	NS	0.0029	0.0002
Glycogen	0.0745[a]	0.0010	0.0426	NS	0.0162
Cytomegaly	0.0009	NS	NS	0.0001	0.0001
CA	—	NS	0.0031	0.0001	0.0001
Steatosis	NS	NS	0.0202[b]	0.0004[b]	0.0001[b]
Zonal change	NS	NS	NS	0.0001	0.0002
Atypical nuclei	NS	0.0019	0.0994[a]	0.0001	0.0001
Enlarged nuclei	NS	0.0901[a]	NS	0.0005	NS

NS, not significant. Linear trends were considered in the analysis. The interim sacrifices included 10 animals/group with variable numbers of animals at the 100-week sacrifice.
[a]p-Values with marginal significance. [b]Negative correlation.

AHF, indicating a faster growth rate of these AHF compared to eosinophilic AHF. This was consistent with the study of Stauber and Bull (1997), which treated male B6C3F$_1$ mice with 2 g/L DCA for 38 or 50 weeks before transferring groups to 0 to 2 g/L DCA for another 2 weeks. Small AHF (> 300 cells/AHF) were primarily eosinophilic, while the large AHF (> 1,000 cells/focus and corresponding to the LFCA) were primarily basophilic. The population of neoplastic lesions was found to have a significantly higher ratio of basophilic to eosinophilic lesions (Stauber and Bull 1997), which is similar to the findings in the present study. As seen in Tables 3 and 4, the number of basophilic and/or clear cell LFCA and ADs per animal was significantly different from controls at both low and high doses of DCA, and the development of basophilic and/or clear cell AHF, LFCA, and ADs was related to length of exposure and DCA dose. We interpret the data in Figure 6 as showing the faster growth rate of basophilic and/or clear cell lesions and time dependence of the transformations between lesions. Thus, at both lower and higher doses of DCA, the number of basophilic and/or clear cell AHF per mouse (the smallest lesions) increased until 78 weeks. At 100 weeks the number of basophilic and/or clear cell AHF per mouse decreased sharply, and this decrease corresponded to a sharp increase in the larger lesions, the basophilic and/or clear cell LFCA. In contrast, the number of eosinophilic AHF per mouse increased with increasing length of exposure, while significant numbers of the larger lesions, eosinophilic LFCA, never appear (Figure 6). Thus, DCA promoted clonal expansion of basophilic and/or clear cell lesions. DCA also promoted neoplastic progression of premalignant basophilic and/or clear cell lesions: more than 48% of either the LFCA or ADs with dysplasia or CA were basophilic and/or clear cell (Fig. 2C).

Richmond et al. (1991) previously reported that LFCA in DCA-treated animals contained foci of cells that expressed tumor markers more frequently found in ADs and CAs. Here the number of basophilic and/or clear cell ADs increased at lower DCA doses throughout the study (Figure 6). At higher DCA doses the number of basophilic and/or clear cell ADs per mouse increased sharply at 52 weeks, then plateaued through 100 weeks, while the number of CAs per mouse increased in stepwise fashion at 78 and 100 weeks, suggesting a transformation of the basophilic and/or clear cell ADs to CAs.

The similarity in appearance of atypical cells in non-involved liver early in control and DCA-treated animals to those found in CAs supports the third lesion sequence (from atypical cells to dysplastic AHF and CAs directly). The presence of atypical nuclei in non-involved liver was associated with the number of CAs per mouse even when the data were adjusted for DCA dose and length of exposure. Moreover, logistic regression analysis indicated that the presence of atypical nuclei in the non-involved liver was highly correlated with both DCA dose and length of exposure (Tables 6 and 7). Consistent with these data, Stauber et al. (1998) found that DCA promoted the outgrowth of anchorage-independent colonies from hepatocytes isolated from naive 5- to 8-week old mice above the small number of anchorage-independent colonies that grew out of the population of untreated hepatocytes.

Since the present histopathologic analysis indicates that DCA does not induce a unique type of lesion, DCA may be acting as a non-genotoxic carcinogen in this model. Exposure of mice to DCA induces a multitude of toxic and adaptive hepatic responses including mitoinhibition, peroxisome proliferation, glycogen accumulation, and endocrine disruption (Carter et al. 1995a; DeAngelo et al. 1999; Kato-Weinstein et al. 1998). Here we examined the relationship of some of these toxic and adaptive responses to DCA dose and length of exposure and to neoplastic progression. A strong correlation between the development of CAs and early liver weight gain (before the development of tumors) was found in these animals with increasing DCA dose (DeAngelo et al. 1999). The liver weight was a reflection of the hepatomegaly that resulted in part from accumulation of glycogen in hepatocytes (Carter et al. 1995a; International Life Sciences Institute 1997; Stauber et al. 1998). The incidence of glycogen accumulation was dose dependent. In mice exposed to 0.5 g/L DCA, glycogen accumulation was seen only in the periportal hepatocytes. At concentrations ≥ 1 g/L, glycogen accumulation was diffuse and occupied greater than 50% of the lobules.

The degree to which hepatocellular necrosis underlies the carcinogenic process is not fully understood, but could be significant

Table 7. Logistic regression analysis (*p*-values) of incidence of toxic or adaptive responses with length of exposure at given doses of DCA and overall adjusted for dose (Overall adj).

Toxic/adaptive response	Control	0.5 g/L	1.0 g/L	2.0 g/L	3.5 g/L	Overall adj
Necrosis	NS	—	0.0021[a]	0.0145[a]	NS	0.0024[a]
Glycogen	—	—	0.0195	NS	NS	0.0001[a]
Cytomegaly	—	NS	0.0371[a]	0.0991[a,b]	0.0910[a,b]	0.0027[a]
CA	—	—	0.0345	0.0006	0.0010	0.0001
Steatosis	0.0773[b]	0.0074	0.0350	NS	NS	0.0001
Zonal change	NS	0.0711[b]	0.0002[a]	0.0197[a]	NS	0.0038
Atypical nuclei	0.0302	0.0101	0.0001	0.0002	0.0222	0.0001
Enlarged nuclei	NS	0.0375	0.0001	0.0004	0.0016	0.0001

NS, not significant. Controls received 0 g/L DCA. Linear trends were considered in the analysis. The interim sacrifices included 10 animals/group with variable numbers of animals at the 100-week sacrifice.
[a]Negative correlation. [b]*p*-Values with marginal significance.

Table 8. Summary of recent *in vitro* and *in vivo* genotoxicity assays for dichloroacetic acid.

	Activity	Reference
In vitro assays		
DNA alkaline unwinding assay		Chang et al. 1992
Rat hepatocytes (0.128–1.28 g/L, 4 hr)[a]	Not active	
Mouse hepatocytes (0.013–1.28 g/L, 4 hr)[a]	Not active	
Prophage-induction assay (+S9; ~2 g/L)[b]	Positive	DeMarini et al. 1994
Salmonella T-100 assay (0.05 g/L)[c]	Positive	DeMarini et al. 1994
L5178Y/TK$^{+/-}$–3.7.2C Mouse lymphoma assay (0.8 g/L)[d]	Positive	Harrington-Brock et al. 1998
In vivo assays		
DNA alkaline unwinding assay		Chang et al. 1992
Mouse liver (0.64 g/kg, 4 hr)[e]	Positive	
Mouse liver (0.5 and 5 g/L, 7 and 14 days exposure)	Not active	
Rat liver (0.128–1.28 g/kg, 4 hr)	Not active	
Rat liver (2 g/L, 30 weeks exposure)	Not active	
Ras oncogene activation (mouse CA, 1 and 3.5 g/L, 100 weeks exposure)[f]	Not active	Ferreira-Gonzalez et al. 1995
Mouse peripheral blood micronucleus assay		Fuscoe et al. 1997
MN-PCE (3.5 g/L, 9 days exposure)[g]	Positive	
MN-PCE (3.5 g/L, 28 days exposure)[h]	Positive	
Single cell gel assay (3.5 g/L, 28 days exposure)[i]	Positive	
LacI transgenic mouse liver (1 and 3.5 g/L, 4 and 10 weeks)[j]	Negative	Leavitt et al. 1997

[a]Concentration range, 4-hr treatment. [b]Lowest effective concentration that produced a 3-fold increase in plaque-forming units/plate relative to the background. [c]Lowest effective concentration that produced a 2-fold increase in reversions/plate relative to the background. [d]Concentration required to give a response equal to 0.013 g/L methylmethanesulfonate. [e]Lowest dose producing significant DNA damage (1.08-fold). [f]No increase in the percent *ras* mutations in DCA-induced tumors when compared to tumors from untreated mice. [g]Lowest DCA concentration giving a positive response (1.8-fold). [h]Lowest DCA concentration giving a positive response (1.3-fold). [i]Lowest DCA concentration giving a positive response. [j]No increased *lacI* mutations in the livers from DCA treated mice when compared to livers from control animals.

at higher DCA concentrations (≥ 1g/L). The extent of necrosis in animals exposed to 0.5 g/L measured both in this study and in a previous analysis (International Life Sciences Institute 1997) was confined to individual hepatocyes and small clusters of cells. The overall prevalence of both glycogen accumulation and necrosis was less than the overall prevalence of CAs (Table 5). Whereas dysplastic AHF, dysplastic LFCA, and dysplastic ADs were not associated with necrosis, dysplastic ADs were associated with glycogen accumulation. Overall, both glycogen accumulation and necrosis were negatively correlated with length of DCA exposure (Table 7), indicating adaptation in non-involved hepatocytes.

DCA also resulted in adaptive changes in lipid metabolism in the non-involved hepatocytes of mice chronically exposed to DCA, as evidenced by an inhibition of steatosis (lipidosis) (Table 6). Steatosis was negatively associated with both dysplastic LFCA and CA. Presumably, many other alterations in hepatic metabolism occur in DCA-treated mice as a result of endocrine disruption. Moore and DeAngelo (1997) reported that, during DCA-induced carcinogenesis, both serum corticosteroid levels and hepatic 11β-hydroxysteroid dehydrogenase (the enzyme that catalyses the conversion of corticosterone to 11-deoxycorticosterone) were increased in a dose- and time-dependent manner. At the same time that DCA altered serum corticosterone levels, it also altered the binding activity and subcellular localization of the cytoplasmic and nuclear glucocortcoid receptor in mouse liver (DeAngelo and McFadden 1995).

Generalized hepatocyte mitoinhibition was characteristic of DCA treatment (Carter et al. 1995a; DeAngelo 2000a; Stauber and Bull 1997). Early mitoinhibition was both dose- and time-dependent *in vivo* and was a direct effect of DCA because it occurred in isolated hepatocytes *in vitro* (Carter et al. 1995a; DeAngelo 2000a). Mitoinhibition was accompanied by a suppression in spontaneous apoptosis in non-involved hepatocytes (Snyder et al. 1995). By selective toxicity on normal hepatocytes, DCA may be promoting the outgrowth of cells either resistant to mitoinhibition (basophilic AHF) or able to metabolize DCA to non-mitoinhibitory products (eosinophilic AHF). The presence of necrosis could further enhance clonal expansion of cells resistant to DCA mitoinhibition. Recent data indicated that dose-dependent DCA-induced mitoinhibition was also found in premalignant hepatic lesions as well as a progressive dysregulation of p39 c-*jun* expression (Carter et al. 2001a, 2001b).

When considered together, these data suggest that DCA acts to alter the homeostasis of hepatocytes, leading to negative selection of cells with a new state of differentiation resistant to DCA toxicity. These effects of DCA are dose dependent and occur at concentrations < 1 g/L, which is the inflection point of the dose–response curve for carcinogenesis. DCA-induced suppression of apoptosis, the natural process for eliminating initiated cells, would increase the growth and/or survival of these preneoplastic cells and lesions. The strong associations found here between the occurrence of atypical cells in non-involved liver and dysplastic LFCA ($p = 0.0001$), dysplastic AD ($p = 0.0074$) and CA ($p = 0.0136$) are consistent with this conclusion.

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